PHARMACEUTICAL COMPOSITION COMPRISING AN IMMUNOGLOBULIN FC REGION AS A CARRIER

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References Cited
U.S. PATENT DOCUMENTS

5,349,053 A 9/1994 Landolfi .......... 530/351
5,455,165 A 10/1995 Capon et al. .......... 424/133.1
5,514,582 A 5/1996 Capon et al. .......... 424/133.1
5,585,097 A 12/1996 Bolt et al. .......... 424/133.1
5,605,690 A 2/1997 Jacobs et al. .......... 424/133.1
5,650,150 A 7/1997 Gillies .......... 530/391.7
5,714,147 A 2/1998 Capon et al. .......... 424/133.1

5,843,725 A 12/1998 Sledziewski et al. .......... 424/134.1
5,908,626 A 6/1999 Chang et al. .......... 424/134.1
5,977,310 A 11/1999 Namiki et al. .......... 530/351
6,030,613 A 2/2000 Blumberg et al. .......... 424/134.1
6,117,656 A 9/2000 Seid .......... 424/134.1
6,165,476 A 12/2000 Strom et al. .......... 424/134.1
6,277,375 B1 8/2001 Ward .......... 424/134.1
6,300,099 B1 10/2001 Sledziewski et al. .......... 424/134.1
6,223,323 B1 11/2001 Sledziewski et al. .......... 424/134.1
6,340,742 B1 1/2002 Burg et al. .......... 530/351
6,410,008 B1 6/2002 Strom et al. .......... 424/85.2
6,482,919 B1 11/2002 Ledbetter et al. .......... 424/185.1
6,541,610 B1 4/2003 Smith .......... 424/185.1
6,620,413 B1 9/2003 DeSauvage et al. .......... 424/185.1
6,656,728 B1 1/2003 Kavamura et al. .......... 424/185.1

FURTHER REFERENCES

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ABSTRACT

Disclosed is a novel use of an immunoglobulin Fc fragment, and more particularly, a pharmaceutical composition comprising an immunoglobulin Fc fragment as a carrier. The pharmaceutical composition comprising an immunoglobulin Fc fragment as a carrier remarkably extends the serum half-life of a drug while maintaining the in vivo activity of the drug at relatively high levels. Also, when the drug is a polypeptide drug, the pharmaceutical composition has less risk of inducing immune responses compared to a fusion protein of the immunoglobulin Fc fragment and a target protein, and is thus useful for developing long-acting formulations of various polypeptide drugs.

13 Claims, 18 Drawing Sheets
### U.S. PATENT DOCUMENTS

<table>
<thead>
<tr>
<th>Patent Number</th>
<th>Application Date</th>
<th>Inventors</th>
</tr>
</thead>
<tbody>
<tr>
<td>7,125,843 B2</td>
<td>10/2006</td>
<td>DeFrees et al.</td>
</tr>
</tbody>
</table>

### FOREIGN PATENT DOCUMENTS

<table>
<thead>
<tr>
<th>Country</th>
<th>Application Number</th>
<th>Application Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>WO</td>
<td>9700319 A2</td>
<td>1/1997</td>
</tr>
<tr>
<td>WO</td>
<td>97724137 A1</td>
<td>7/1997</td>
</tr>
<tr>
<td>WO</td>
<td>9902709 A1</td>
<td>1/1999</td>
</tr>
<tr>
<td>WO</td>
<td>0040615 A2</td>
<td>2/2000</td>
</tr>
<tr>
<td>WO</td>
<td>0069913 A1</td>
<td>1/2000</td>
</tr>
<tr>
<td>WO</td>
<td>0103737 A1</td>
<td>1/2001</td>
</tr>
<tr>
<td>WO</td>
<td>02066514 A2</td>
<td>8/2002</td>
</tr>
<tr>
<td>WO</td>
<td>2004016739 A2</td>
<td>11/2004</td>
</tr>
<tr>
<td>WO</td>
<td>200401740 A2</td>
<td>11/2004</td>
</tr>
<tr>
<td>WO</td>
<td>2004058885 A2</td>
<td>12/2004</td>
</tr>
<tr>
<td>WO</td>
<td>2005001025 A2</td>
<td>1/2005</td>
</tr>
<tr>
<td>WO</td>
<td>2005017148 A1</td>
<td>2/2005</td>
</tr>
<tr>
<td>WO</td>
<td>2005037989 A2</td>
<td>4/2005</td>
</tr>
<tr>
<td>WO</td>
<td>2005047346 A1</td>
<td>5/2005</td>
</tr>
<tr>
<td>WO</td>
<td>2005073383 A3</td>
<td>8/2005</td>
</tr>
</tbody>
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* cited by examiner
FIG. 1

Protein A (stepwise pH elution)
FIG. 2

M IgG Fc
FIG. 3

A) IFNα

B) V7Ser-G-CSF

C) EPO

M: Molecular size marker
Lane 1: Fc
Lane 2: Physiologically active protein
Lane 3: Physiologically active protein-PEG-Fc conjugate
FIG. 6b

M  Fc  DG Fc
FIG. 8b
FIG. 8c
Fig. 9

![Graph showing concentration over time for different compounds.](image)
PHARMACEUTICAL COMPOSITION COMPRISING AN IMMUNOGLOBULIN Fc REGION AS A CARRIER

TECHNICAL FIELD

The present invention relates to a novel use of an immunoglobulin Fc fragment. More particularly, the present invention relates to a pharmaceutical composition comprising an immunoglobulin Fc fragment as a carrier, and a method of improving the in vivo duration of action of a drug linked to an immunoglobulin Fc fragment.

BACKGROUND ART

In the past, a large number of pharmacologists and chemists have made efforts to chemically alter and/or modify the in vivo activity of naturally occurring, physiologically active molecules. These efforts mainly focused on increasing or prolonging certain in vivo activity, reducing toxicity, eliminating or reducing side effects, or modifying specific physiological activities of the physiologically active substances. When a physiologically active substance is chemically modified, it loses some or most of its physiological activities in many cases. However, in some cases, the modification could result in an increase or change in physiological activity. In this regard, many studies have been focused on chemical modification capable of achieving desired physiological activity, and most of such studies have involved covalently bonding a physiologically active substance (drug) to a physiologically acceptable carrier.

For example, International Patent Publication No. WO 01/93911 employs a polymer having a plurality of acid moieties as a drug carrier. International Patent Publication No. WO 03/00778, discloses an anionic group-containing amphiphilic block copolymers that, when used as a drug carrier for a cationic drug, improve the stability of the drug. European Patent No. 0681481 describes a method of improving the properties of basic drugs by using cyclodextrin and acids as carriers. On the other hand, hydrophobic drugs have low stability in vivo mainly due to their low aqueous solubility. To improve the low aqueous solubility of hydrophobic drugs, International Patent Publication No. WO 04/064731 employs a lipid as a carrier. However, to date, there is no report for the use of an immunoglobulin Fc fragment as a drug carrier.

Typically, since polypeptides are relatively easily denatured due to their low stability, degraded by proteolytic enzymes in the blood and easily eliminated through the kidney or liver, protein medicaments, including polypeptides as pharmaceutically effective components, need to be frequently administered to patients to maintain desired blood level concentrations and titers. However, this frequent administration of protein medicaments, especially through injection causes pain for patients. To solve these problems, many efforts have been made to improve the serum stability of protein drugs and maintain the drugs in the blood at high levels for a prolonged period of time, and thus maximize the pharmaceutical efficacy of the drugs. Pharmaceutical compositions with sustained activity, therefore, need to increase the stability of protein drugs and maintain the titers at sufficiently high levels without causing immune responses in patients.

To stabilize proteins and prevent enzymatic degradation and clearance by the kidneys, a polymer having high solubility, such as polyethylene glycol (hereinafter, referred to simply as “PEG”), was conventionally used to chemically modify the surface of a protein drug. By binding to specific or various regions of a target protein, PEG stabilizes the protein and prevents hydrolysis, without causing serious side effects (Sada et al., J. Fermentation Bioengineering 71: 137-139, 1991). However, despite its capability to enhance protein stability, this PEG coupling has problems such as greatly reducing the number of interactions of physiologically “active” proteins. Further, the yield decreases with the increasing molecular weight of PEG due to the reduced reactivity with the proteins.

Recently, polymer-protein drug conjugates have been suggested. For example, as described in U.S. Patent No. 5,738,846, a conjugate can be prepared by linking an identical protein drug to both ends of PEG to improve the activity of the protein drug. Also, as described in International Patent Publication No. WO 92/16221, two different protein drugs can be linked to both ends of PEG to provide a conjugate having two different activities. The above methods, however, were not very successful in sustaining the activity of protein drugs.

On the other hand, Kinstler et al. reported that a fusion protein prepared by coupling granulocyte-colony stimulating factor (G-CSF) to human albumin showed improved stability (Kinstler et al., Pharmaceutical Research 12(12): 1883-1888, 1995). In this publication, however, since the modified drug, having a G-CSF-PEG-albumin structure, only showed an approximately four-fold increase in residence time in the body and a slight increase in serum half-life compared to the single administration of the native G-CSF, it has not been industrialized as an effective long-acting formulation for protein drugs.

An alternative method for improving the in vivo stability of physiologically active proteins is by linking a gene of physiologically active protein to a gene encoding a protein having high serum stability by genetic recombination technology and culturing the cells transfected with the recombinant gene to produce a fusion protein. For example, a fusion protein can be prepared by conjugating albumin, a protein known to be the most effective in enhancing protein stability, or its fragment to a physiologically active protein of interest by genetic recombination (International Patent Publication Nos. WO 93/15119 and WO 93/15200, European Patent Publication No. 413,622). A fusion protein of interferon-alpha and albumin, developed by the Human Genome Science Company and marketed under the trade name of “Albuferon™,” increased the half-life from 5 hours to 93 hours in monkeys, but it was known to be problematic because it decreased the in vivo activity to less than 5% of unmodified interferon-alpha (Osborn et al., J. Pharm. Exp. Ther. 303(2): 540-548, 2002).

Recombinant DNA technologies were applied to fuse a protein drug to an immunoglobulin Fc fragment. For example, interferon (Korean Patent. Lay-open Publication No. 2003-9464), and interleukin-4 receptor, interleukin-7 receptor or erythropoietin (EPO) receptor (Korean Patent Registration No. 249572) were previously expressed in mammals in a form fused to an immunoglobulin Fc fragment. International Patent Publication No. WO 01/07373 describes a fusion protein comprising a cytokine or growth factor linked to an immunoglobulin Fc fragment through peptide linkage. In addition, U.S. Patent No. 5,116,964 discloses proteins fused to the amino or carboxyl-terminal end of an immunoglobulin Fc fragment by genetic recombination. U.S. Patent No. 5,349,053 discloses a fusion protein comprising IL-2 fused to an immunoglobulin Fc fragment through peptide linkage. Other examples of fusion proteins prepared by genetic recombination include a fusion protein of interferon-beta or its derivative and an immunoglobulin Fc fragment (International Patent Publication No. WO 00/23472), and a fusion protein of IL-5 receptor and an immunoglobulin Fc fragment (U.S. Patent No. 5,712,121), a fusion protein of interferon alpha and the Fc fragment of...
immunoglobulin G4 (U.S. Pat. No. 5,723,125); and a fusion protein of CD4 protein and the Fc fragment of immunoglobulin G2 (U.S. Pat. No. 6,451,313).

Techniques involving the modification of amino acid residues of an immunoglobulin Fc fragment are also known. For example, U.S. Pat. No. 5,605,690 discloses a TNFR-IgG1 Fc fusion protein, which is prepared by genetic recombination using an IgG1 Fc fragment having amino acid alterations in the complement binding region or receptor binding region. Also, other methods of preparing a fusion protein using a modified immunoglobulin Fc fragment by genetic recombination are disclosed in U.S. Pat. Nos. 6,277,375, 6,410,008 and 6,444,792.

Such Fc fusion proteins produced by genetic recombination have the following disadvantages: protein fusion occurs only in a specific region of an immunoglobulin Fc fragment, which is at an amino- or carboxyl-terminal end; only homodimeric forms and not monomeric forms are produced; and a fusion could take place only between the glycosylated proteins or between the aglycosylated proteins, and it is impossible to make a fusion protein composed of a glycosylated protein and an aglycosylated protein. Further, a new amino acid sequence created by the fusion may trigger immune responses, and a linker region may become susceptible to proteolytic degradation.

To solve these problems, the inventors of the present application conducted research, and came to a knowledge that, when a drug is administered in the form of being linked to an IgG Fc fragment, the drug has improved in vivo stability while exhibiting a minimal reduction in the in vivo activity.

**DISCLOSURE OF THE INVENTION**

It is therefore an object of the present invention to provide a pharmaceutical composition comprising an immunoglobulin Fc fragment as a carrier. It is another object of the present invention to provide a method for improving the in vivo duration of action of a drug by including an immunoglobulin Fc fragment as a carrier.

**BRIEF DESCRIPTION OF THE DRAWINGS**

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

FIG. 1 shows the results of chromatography of an immunoglobulin Fc fragment obtained by cleavage of an immunoglobulin with papain;

FIG. 2 shows the results of SDS-PAGE of a purified immunoglobulin Fc fragment (M: molecular size marker, lane 1: IgG, lane 2: Fc);

FIG. 3 shows the results of SDS-PAGE of IFNcc-PEG-Fc (A), 15Ser-G-CSF-PEG-Fc (B) and EPO-PEG-Fc (C) conjugates, which are generated by a coupling reaction (M: molecular size marker, lane 1: Fc, lane 2: physiologically active protein, lane 3: physiologically active protein-PEG-Fc conjugate);

FIG. 4 shows the results of size exclusion chromatography of an IFNcc-PEG-Fc conjugate that is purified after a coupling reaction;

FIG. 5 shows the results of MALDI-TOF mass spectrometry of an EPO-PEG-Fc conjugate;

FIGS. 6a and 6b show the results of MALDI-TOF mass spectrometry and SDS-PAGE analysis, respectively, of a native immunoglobulin Fc and a deglycosylated immunoglobulin Fc (DG Fc);

FIG. 7 shows the results of MALDI-TOF mass spectrometry of an IFNcc-PEG-Fc conjugate and an IFNcc-PEG-DG Fc conjugate;

FIGS. 8a to 8c show the results of reverse phase HPLC of IFNcc-PEG-Fc, IFNcc-PEG-DG Fc and IFNcc-PEG-recombinant AG Fc derivative conjugates;

FIG. 9 is a graph showing the results of pharmacokinetic analysis of a native IFNcc, an IFNcc-40K PEG complex, an IFNcc-PEG-albumin conjugate and an IFNcc-PEG-Fc conjugate;

FIG. 10 is a graph showing the results of pharmacokinetic analysis of a native EPO, a highly glycosylated EPO, an EPO-PEG-Fc conjugate and an EPO-PEG-AG Fc conjugate;

FIG. 11 is a graph showing the results of pharmacokinetic analysis of IFNcc-PEG-Fc, IFNcc-PEG-DG Fc and IFNcc-PEG-recombinant AG Fc conjugates;

FIG. 12 is a graph showing the pharmacokinetics of a Fabβ, a Fabβ-S-40K PEG complex, a Fabβ-N-PEG-N-Fc conjugate and a Fabβ-S-PEG-N-Fc conjugate;

FIG. 13 is a graph showing the in vivo activities of Fabβ, a Fabβ-S-40K PEG complex, a Fabβ-N-PEG-N-Fc conjugate and a Fabβ-S-PEG-N-Fc conjugate;

FIG. 14 is a graph showing the results of comparison of human IgG subclasses for binding affinity to the C1q complement;

FIG. 15 is a graph showing the results of comparison of a glycosylated Fc, an enzymatically deglycosylated DG Fc and an interferon-PEG-carrier conjugate where the carrier is AG Fc produced by E. coli for binding affinity to the C1q complement.

**BEST MODE FOR CARRYING OUT THE INVENTION**

In one aspect, the present invention relates to a pharmaceutical composition comprising an immunoglobulin Fc fragment as a carrier.

The term “carrier”, as used herein, refers to a substance linked with a drug, which typically increases, decreases or eliminates the physiological activity of the drug by binding to the drug. However, with respect to the objects of the present invention, a carrier is employed in the present invention for minimizing a decrease in the physiological activity of a drug of interest, linked to the carrier, while enhancing the in vivo stability of the drug.

To accomplish the objects of the present invention, the present invention is characterized by employing an immunoglobulin Fc fragment as a carrier.

The immunoglobulin Fc fragment is safe for use as a drug carrier because it is a biodegradable polypeptide that is metabolized in the body. Also, the immunoglobulin Fc fragment has a relatively low molecular weight compared to the whole immunoglobulin molecule, thus being advantageous in the preparation, purification and yield of conjugates due to. Further, since the immunoglobulin Fc fragment does not contain the Fab fragment, whose amino acid sequence differs among antibody subclasses and which thus is highly non-homogenous, it may greatly increase the homogeneity of substances and be less antigenic.

The term “immunoglobulin Fc fragment”, as used herein, refers to a protein that contains the heavy-chain constant region 2 (C\text{\textsubscript{2}}) and the heavy-chain constant region 3 (C\text{\textsubscript{3}}) of an immunoglobulin, and not the variable regions of the heavy and light chains, the heavy-chain constant region 1 (C\text{\textsubscript{1}}) and the light-chain constant region 1 (C\text{\textsubscript{L}}) of the immunoglobulin. It may further include the hinge region at the heavy-chain constant region. Also, the immunoglobulin
Fc fragment of the present invention may contain a portion or the all the heavy-chain constant region 1 (C₃h1) and/or the light-chain constant region 1 (C₃l1), except for the variable regions of the heavy and light chains. Also, as long as it has a physiological function substantially similar to or better than the native protein the IgG Fc fragment may be a fragment having a deletion in a relatively long portion of the amino acid sequence of C₃h2 and/or C₃l2. That is, the immunoglobulin Fc fragment of the present invention may comprise 1) a C₃h1 domain, a C₃h2 domain, a C₃l3 domain and a C₃l4 domain, 2) a C₃h1 domain and a C₃h2 domain, 3) a C₃h1 domain and a C₃l3 domain, 4) a C₃h2 domain and a C₃l3 domain, 5) a combination of one or more domains and an immunoglobulin hinge region (or a portion of the hinge region), and 6) a dimer of each domain of the heavy-chain constant regions and the light-chain constant region.

The Fc fragment of the present invention includes a native amino acid sequence and sequence derivatives (mutants) thereof. An amino acid sequence derivative is a sequence that is different from the native amino acid sequence due to a deletion, an insertion, a non-conservative or conservative substitution or combinations thereof of one or more amino acid residues. For example, in an IgG Fc, amino acid residues known to be important in binding, at positions 214 to 238, 297 to 299, 318 to 322, or 327 to 331, may be used as a suitable target for modification. Also, other various derivatives are possible, including one in which a region capable of forming a disulfide bond is deleted, or certain amino acid residues are eliminated at the N-terminal end of a native Fc form or a methionine residue is added thereto. Further, to remove effector functions, a deletion may occur in a complement-binding site, such as a C1q-binding site and an ADCC site. Techniques of preparing such sequence derivatives of the immunoglobulin Fc fragment are disclosed in International Patent Publication Nos. WO 97/34631 and WO 96/32478.

Amino acid exchanges in proteins and peptides, which do not generally alter the activity of the proteins, or peptides are known in the art (H. Neurath, R. L. Hill, The Proteins, Academic Press, New York, 1979). The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Thr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu and Asp/Gly, in both directions.

In addition, the Fc fragment, if desired, may be modified by phosphorylation, sulfation, acylation, glycosylation, methylation, formylation, acetylation, amidation, and the like.

The aforementioned Fc derivatives are derivatives that have a biological activity identical to the Fc fragment of the present invention or improved structural stability, for example, against heat, pH, or the like.

In addition, these Fc fragments may be obtained from native forms isolated from humans and other animals including cows, goats, swine, mice, rabbits, hamsters, rats and guinea pigs, or may be recombinants or derivatives thereof, obtained from transformed animal cells or microorganisms. Herein, they may be obtained from a native immunoglobulin by isolating whole immunoglobulins from human or animal organisms and treating them with a proteolytic enzyme. Papain digests the native immunoglobulin into Fab and Fc fragments, and pepsin treatment results in the production of pFc' and F' (ab')2 fragments. These fragments may be subjected, for example, to size exclusion chromatography to isolate Fc or pFc'.

Preferably, a human-derived Fc fragment is a recombinant immunoglobulin Fc fragment that is obtained from a microorganism.
The term “drug,” as used herein, refers to a substance displaying therapeutic activity when administered to humans or animals, and examples of the drug include, but are not limited to, polypeptides, compounds, extracts and nucleic acids. Preferred is a polypeptide drug.

The term “physiologically active polypeptide”, “physiologically active protein”, “active polypeptide” “polypeptide drug” and “protein drug”, as used herein, are interchangeable in their meanings, and are featured in that they are in a physiologically active form exhibiting various in vivo physiological functions.

The polypeptide drug has a disadvantage of being unable to sustain physiological action for a long period of time due to its property of being easily denatured or degraded by proteolytic enzymes present in the body. However, when the polypeptide drug is conjugated to the immunoglobulin Fc fragment of the present invention to form a conjugate, the drug has increased structural stability and degradation half-life. Also, the polypeptide conjugated to the Fc fragment has a much smaller decrease in physiological activity than other known polypeptide drug formulations. Therefore, compared to the in vivo bioavailability of conventional polypeptide drugs, the conjugate of the polypeptide and the immunoglobulin Fc fragment according to the present invention is characterized by having markedly improved in vivo bioavailability. This is also clearly described through embodiments of the present invention. That is, when linked to the immunoglobulin Fc fragment of the present invention, IFNα, G-CSF, HGH and other protein drugs displayed an about two- to six-fold increase in vivo bioavailability compared to their conventional forms conjugated to PEG alone or both PEG and albumin (Tables 8, 9 and 10).

On the other hand, the linkage of a protein and the immunoglobulin Fc fragment of the present invention is featured in that it is not a fusion by a conventional recombination method. A fusion form of the immunoglobulin Fc fragment and an active polypeptide used as a drug by a recombination method is obtained in such a way that the polypeptide is linked to the N-terminus or C-terminus of the Fc fragment, and is thus expressed and folded as a single polypeptide from a nucleotide sequence encoding the fusion form.

This brings about a sharp decrease in the activity of the resulting fusion protein because the activity of a protein as a physiologically functional substance is determined by the conformation of the protein. Thus, when a polypeptide drug is fused with Fc by a recombination method, there is no effect with regard to in vivo bioavailability even when the fusion protein has increased structural stability. Also, since such a fusion protein is often misfolded and thus expressed as inclusion bodies, the fusion method is uneconomical in protein production and isolation yield. Further, when the active form of a polypeptide is in a glycosylated form, the polypeptide should be expressed in eukaryotic cells. In this case, Fc is also glycosylated, and this glycosylation may cause unsuitable immune responses in vivo.

That is, only the present invention makes it possible to produce a conjugate of a glycosylated active polypeptide and an aglycosylated immunoglobulin Fc fragment, and overcomes all of the above problems, including improving protein production yield, because the two components of the complex are individually prepared and isolated by the best systems.

Non-limiting examples of protein drugs capable of being conjugated to the immunoglobulin Fc fragment of the present invention include human growth hormone, growth hormone releasing hormone, growth hormone releasing peptide, interferons and interferon receptors (e.g., interferon-α, -β and -γ, water-soluble type I interferon receptor, etc.), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), glucagon-like peptides (e.g., GLP-1, etc.), G-protein-coupled receptor, interleukins (e.g., interferon-γ, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -12, -13, -14, -15, -16, -17, -18, -19, -20, -21, -22, -23, -24, -25, -26, -27, -28, -29, -30, etc.) and interleukin receptors (e.g., IL-1 receptor, IL-4 receptor, etc.), enzymes (e.g., glucocerebrosidase, iduronate-2-sulfatase, alpha-galactosidase-A, alpha-galactosidase alpha and beta, alpha-L-iduronidase, butyrylcholinesterase, chitinase II, glutamate decarboxylase, imiglucerase, lipase, uricase, platelet-activating factor acetylhydrolase, neutral endopeptidase, myeloperoxidase, etc.), interleukin and cytokine binding proteins (e.g., IL-18Bp, TNF-binding protein, etc.), macrophage activating factor, macrophage peptide, B cell factor, T cell factor, protein A, allergy inhibitor, cell necrosis glycoproteins, immunotoxins, lymphotoxin, tumor necrosis factor, tumor suppressors, metastasis growth factor, alpha-1 antitrypsin, albumin, alpha-lactalbumin, apolipoprotein-E, erythropoietin, highly glycosylated erythropoietin, angiotensins; hemoglobin, thrombin, thrombin receptor activating peptide, thrombomodulin, factor VII, factor VIIa, factor VIII, factor IX, factor XIII, plasminogen activating factor, fibrin-binding peptide, urokinease, streptokinase, hirudin, protein C, C-reactive protein, renin inhibitor, collagenase inhibitor, superoxide dismutase, leptin, platelet-derived growth factor, epithelial growth factor, epidermal growth factor, angiotatin, angiotensin, bone growth factor, bone stimulating protein, calcitonin, insulin, atriopeptin, cartilage inducing factor, elcatonin, connective tissue activating factor, tissue factor pathway inhibitor, follicle stimulating hormone, luteinizing hormone, luteinizing hormone releasing hormone, nerve growth factors (e.g., nerve growth factor, ciliary neurotrophic factor, axogenesis factor-1, brain-natriuretic peptide, galil derived neurotrophic factor, neurtin, neurophil inhibitor factor, neurotrophic factor, neurotensin, etc.), parathyroid hormone, relaxin, secretin, somatomedin, insulin-like growth factor, adenocortical hormone, glucagon, cholecystokinin, pancreatic polypeptide, gastrin releasing peptide, corticotropin releasing factor, thyroid stimulating hormone, autotaxin, leucotrienn, myostatin, receptors (e.g., TNRF(P75), TNRF(P55), IL-1 receptor, VEGF receptor, B cell activating factor receptor, etc.), receptor antagonists (e.g., IL-1-Ra etc.), cell surface antigens (e.g., CD 2, 3, 4, 5, 7, 11a, 11b, 18, 19, 20, 23, 25, 33, 38, 40, 45, 69, etc.), virus vaccine antigens, monocolonal antibodies, polyclonal antibodies, antibody fragments (e.g., scFv, Fab, Fab’, Fab(αb)2 and Fd), and virus derived vaccine antigens. An antibody fragment may be Fab, Fab’, F(ab’)2, Fd or scFv, which is capable of binding to a specific antigen, and preferably Fab’. TheFab’ fragments contain the variable domain (Vj) and constant domain (Cj) of the light chain and the variable domain (Vj) and the first constant domain (Cj),1 of the heavy chain. TheFab’ fragments differ from the Fab fragments in terms of adding several amino acid residues including one or more cysteine residues from the hinge region to the carboxyl terminal of the Cj domain. The Fd fragments comprise only the VH and Cβ1 domain, and the F(ab’)2 fragments are produced as a pair of Fab’ fragments by either disulfide bonding or a chemical reaction. The scFv (single-chain Fv) fragments comprise the Vj and Vj domains that are linked to each other by a peptidyl linker and thus are present in a single polypeptide chain.

In particular, preferred as physiologically active polypeptides are those requiring frequent dosing upon administration to the body for therapy or prevention of diseases, which include human growth hormone, interferons (interferon-α, -β, -γ, etc.), granulocyte colony stimulating factor, erythro-
poietin (EPO) and antibody fragments. In addition, certain derivatives are included in the scope of the physiologically active polypeptides of the present invention as long as they have function, structure, activity or stability substantially identical to or improved compared over native forms of the physiologically active polypeptides. In the present invention, the most preferable polypeptide drug is interferon-alpha.

In addition to the polypeptide drugs, other drugs are also available in the present invention. Non-limiting examples of these drugs include antibiotics selected from among derivatives and mixtures of tetracycline, minocycline, doxycycline, ofloxacin, levofloxacin, ciprofloxacin, clarithromycin, erythromycin, cefaclor, cefotaxime, imipenem, penicillin, gentamycin, streptomycin, vancomycin, and the like; antineoplastic agents selected from among derivatives and mixtures of methotrexate, carboplatin, taxol, cisplatin, 5-fluorouracil, doxorubicin, etoposide, paclitaxel, camptotecin, cytosine arabinoside, and the like; anti-inflammatory agents selected from among derivatives and mixtures of indomethacin, ibuprofen, ketoprofen, piroxicam, profenofen, and the like; antiviral agents selected from among derivatives and mixtures of acyclovir and rabavirin; and antibacterial agents selected from among derivatives and mixtures of ketoconazole, itraconazole, fluconazole, amphotericin B and griseofulvin.

On the other hand, the immunoglobulin Fc fragment of the present invention is able to form a conjugate linked to a drug through a linker.

This linker includes peptide and non-peptide linkers. Preferred is a non-peptide linker, and more preferably is a non-peptide polymer.

The term “peptide linker”, as used herein, means amino acids, and preferably 1 to 20 amino acids, which are linearly linked to each other by peptide bonding, and may be in a glycosylated form. With respect to the objects of the present invention, preferably is an aglycosylated form. This peptide linker is preferably a peptide having a repeating unit of Gly and Ser, which is immunologically inactive for T cells.

The term “non-peptide polymer”, as used herein, refers to a bioconjugatable polymer including two or more repeating units linked to each other by a covalent bond excluding the peptide bond. Examples of the non-peptide polymer include poly (ethylene glycol), poly (propylene glycol), copolymers of ethylene glycol and propylene glycol, polyoxyethylene polylco-polyvinyl alcohol, polyacetalides, dextran, polyvinyl ether, biodegradable polymers such as PLA (poly (lactic acid)) and PLAGA (poly (lactic-glycolic acid)), lipid polymers, chitins, and hyaluronic acid. The most preferred is poly (ethylene glycol) (PEG).

The conjugate of the present invention, immunoglobulin Fc fragment-drug or immunoglobulin Fc fragment-linker-drug, is made at various molar ratios. That is, the number of the immunoglobulin Fc fragment and/or linker linked to a single polypeptide drug is not limited. However, preferably, in the drug conjugate of the present invention, the drug and the immunoglobulin Fc fragment are conjugated to each other at a molar ratio of 1:1 to 10:1, and preferably 1:1 to 2:1.

In addition, the linkage of the immunoglobulin Fc fragment of the present invention, a certain linker and a certain drug include all covalent bonds except for a peptide bond formed when the Fc fragment and a polypeptide drug are expressed as a fusion protein by genetic recombination, and all types of non-covalent bonds such as hydrogen bonds, ionic interactions, van der Waals forces and hydrophobic interactions. However, with respect to the physiological activity of the drug, the linkage is preferably made by covalent bonds.

In addition, the immunoglobulin Fc fragment of the present invention, a certain linker and a certain drug may be linked to each other at a certain site of the drug. On the other hand, the immunoglobulin Fc fragment of the present invention and a polypeptide drug may be linked to each other at an N-terminus or C-terminus, and preferably at a free group, and a covalent bond between the immunoglobulin Fc fragment and the drug is easily formed especially at an amino terminal end, an amino group of a lysine residue, an amino group of a histidine residue, or a free cysteine residue.

On the other hand, the linkage of the immunoglobulin Fc fragment of the present invention, a certain linker and a certain drug may be made in a certain direction. That is, the linker may be linked to the N-terminus, the C-terminus or a free group of the immunoglobulin Fc fragment, and may also be linked to the N-terminus, the C-terminus or a free group of the protein drug. When the linker is a peptide linker, the linkage may take place at a certain linking site.

Also, the conjugate of the present invention may be prepared using any of a number of coupling agents known in the art. Non-limiting examples of the coupling agents include 1,1-bis (diazacetyl)-2-phenylethylamine, glutaradehyde, N-hydroxysuccinimide esters such as esters with 4-azidosalicylic acid, imidesters including disuccinimidyl esters such as 3,3'- dithiobis (succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane.

On the other hand, the pharmaceutical composition of the present invention, comprising an immunoglobulin Fc fragment as a carrier, may be administered via a variety of routes. The term “administration”, as used herein, means introduction of a predetermined amount of a substance into a patient by a certain suitable method. The conjugate of the present invention may be administered via any of the common routes, as long as it is able to reach a desired tissue. A variety of modes of administration are contemplated, including intraperitoneally, intravenously, intramuscularly, subcutaneously, intradermally, orally, topically, intranasally, intrapulmonarily and intrarectally, but the present invention is not limited to these exemplified modes of administration. However, since peptides are digested upon oral administration, active ingredients of a composition for oral administration should be coated or formulated for protection against degradation in the stomach. Preferably, the present composition may be administered in an injectable form. In addition, the pharmaceutical composition of the present invention may be administered using a certain apparatus capable of transporting the active ingredients into a target cell.

The pharmaceutical composition comprising the conjugate according to the present invention may include a pharmaceutically acceptable carrier. For oral administration, the pharmaceutically acceptable carrier may include binders, lubricants, disintegrators, excipients, solubilizers, dispersing agents, stabilizers, suspending agents, coloring agents and perfumes. For injectable preparations, the pharmaceutically acceptable carrier may include buffering agents, preserving agents, analgesics, solubilizers, isotonic agents and stabilizers. For preparations for topical administration, the pharmaceutically acceptable carrier may include bases, emulsions, lubricants and preservatives. The pharmaceutical composition of the present invention may be formulated into a variety of dosage forms in combination with the aforementioned pharmaceutically acceptable carriers. For example, for oral administration, the pharmaceutical composition may be formulated into tablets, troches, capsules, elixirs, suspensions, syrups or wafers. For injectable preparations, the pharmaceutical composition may be formulated into a unit dosage form, such as a multidose container or an ampule as a single-
The pharmaceutical composition may be also formulated into solutions, suspensions, tablets, capsules and long-acting preparations.

On the other hand, examples of carriers, exipients and diluents suitable for the pharmaceutical formulations include lactose, dextrose, sucrose, sorbitol, mannitol, xylitol, erythritol, maltitol, starch, acacia rubber, alginate, gelatin, calcium phosphate, calcium silicate, cellulose, methylcellulose, microcrystalline cellulose, polyvinylpyrrolidone, water, methylhydroxybenzoate, propylhydroxybenzoate, t alc, magnesium stearate and mineral oils. In addition, the pharmaceutical formulations may further include fillers, anti-coagulating agents, lubricants, humectants, perfumes, emulsifiers and antisepsic.

A substantial dosage of a drug in combination with the immunoglobulin Fc fragment of the present invention as a carrier may be determined by several related factors including the types of diseases to be treated, administration routes, the patient’s age, gender, weight and severity of the illness, as well as by the types of the drug as an active component. Since the pharmaceutical composition of the present invention has a very long duration of action in vivo, it has an advantage of greatly reducing administration frequency of pharmaceutical drugs.

In another aspect, another object of the present invention is to provide a method of improving the in vivo duration of a drug by comprising an immunoglobulin Fc fragment.

In one embodiment of the present invention, a physiologically active polypeptide-PEG-immunoglobulin Fc fragment conjugate exerts much higher stability than a polypeptide-PEG complex or a polypeptide-PEG-albumin conjugate. Pharmacokinetic analysis revealed that IFNα has a serum half-life increased by about 20 times when linked to 40-kDa PEG (IFNα-40K PEG complex) and about 10 times in an IFNα-PEG-albumin conjugate, compared to the native IFNα. In contrast, an IFNα-PEG-Fc conjugate according to the present invention showed a half-life remarkably increased by about 50 times (see, Table 5). In addition, the same result was observed in other target proteins, human growth hormone (hGH), granulocyte colony-stimulating factor (G-CSF) and its derivative (15S-G-CSF), and erythropoietin (EPO). Protein conjugates according to the present invention, each of which comprises a target protein linked to PEG-Fc, displayed increases about 10-fold in mean residence time (MRT) and serum half-life compared to the native forms of the proteins and the forms conjugated to PEG or PEG-albumin (see, Tables 4 to 7).

In addition, when a PEG-Fc complex is linked to an—SH group near the C-terminus of a Fab or the N-terminus of the Fab, the resulting Fab-Peg-Fc conjugate displayed a 2 to 3-fold longer serum half-life than a 40K PEG-Fab complex (see, FIG. 12). Further, when protein conjugates are prepared using deglycosylated immunoglobulin Fc (DG Fc), where sugar moieties are removed, and recombinant aglycosylated immunoglobulin Fc (Ag Fc) derivatives, their plasma half-lives and in vitro activity were maintained similar to the protein conjugates prepared using the native Fc (see, Table 3 and FIGS. 8 and 11).

Therefore, since the protein conjugates of the present invention have extended serum half-lives and mean residence time (MRT) when applied to a variety of physiologically active polypeptides including human growth hormone, interferon, erythropoietin, colony stimulating factor or its derivatives, and antibody derivatives, they are useful for developing long-acting formulations of diverse physiologically active polypeptides.

A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as the limits of the present invention.

**EXAMPLE 1**

Preparation 1 of IFNα-PEG-immunoglobulin Fc Fragment Conjugate

*<Step 1>* Preparation of Immunoglobulin Fc Fragment using Immunoglobulin

An immunoglobulin Fc fragment was prepared as follows. 200 mg of 150-kDa immunoglobulin G (IgG) (Green Cross, Korea) was dissolved in 10 mM phosphate buffer was treated with 2 mg of a proteolytic enzyme, papain (Sigma) at 37°C for 2 hrs with gentle agitation. After the enzyme reaction, the immunoglobulin Fc fragment regenerated thus was subjected to chromatography for purification using sequentially a Superdex column, a protein A column and a cation exchange column. In detail, the reaction solution was loaded onto a Superdex 200 column (Pharmacia) equilibrated with 10 mM sodium phosphate buffer (pH 7.3), and the column was eluted with the same buffer at a flow rate of 1 ml/min. Unretracted immunoglobulin molecules (IgG) and F(ab')2, which had a relatively high molecular weight compared to the immunoglobulin Fc fragment, were removed using their property of being eluted earlier than the Ig Fc fragment. Fab fragments having a molecular weight similar to the Ig Fc fragment were eliminated by protein A column chromatography (FIG. 1). The resulting fractions containing the Ig Fc fragment eluted from the Superdex 200 column were loaded at a flow rate of 5 ml/min onto a protein A column (Pharmacia) equilibrated with 20 mM phosphate buffer (pH 7.0), and the column was washed with the same buffer to remove proteins unbound to the column. Then, the protein A column was eluted with 100 mM sodium citrate buffer (pH 3.0) to obtain highly pure immunoglobulin Fc fragment. The Fc fractions collected from the protein A column were finally purified using a cation exchange column (polyCAT, Polycl.C Company), wherein this column loaded with the Fc fractions was eluted with a linear gradient of 0.15-0.4 M NaCl in 10 mM acetate buffer (pH 4.5), thus providing highly pure Fc fractions. The highly pure Fc fractions were analyzed by 12% SDS-PAGE (lane 2 in FIG. 2).

*<Step 2>* Preparation of IFNα-PEG complex

3.4-kDa polyethylene glycol having an aldehyde reactive group at both ends, ALD-PEG-ALD (Shearwater), was mixed with human interferon alpha-2b (IFNα-2b, MW: 20 kDa) dissolved in 100 mM phosphate buffer in an amount of 5 mg/ml) at an IFNα: PEG molar ratio of 1:1, 1:2.5, 1:5, 1:10 and 1:20. To this mixture, a reducing agent, sodium cyanoborohydride (NaCNBH₃, Sigma), was added at a final concentration of 20 mM and was allowed to react at 4°C for 3 hrs with gentle agitation to allow PEG to link to the amino terminal end of interferon alpha. To obtain a 1:1 complex of PEG and interferon alpha, the reaction mixture was subjected to size exclusion chromatography using a Superdex 50 column (Pharmacia). The IFNα-PEG complex was eluted from the column using 10 mM potassium phosphate buffer (pH 6.0) as an elution buffer, and interferon alpha not linked to PEG, unreacted PEG and dimer byproducts where PEG was linked to two interferon alpha molecules were removed. The purified IFNα-PEG complex was concentrated to 5 mg/ml. Through this experiment, the optimal reaction molar ratio for IFNα to
PEG, providing the highest reactivity and generating the smallest amount of byproducts such as dimers, was found to be 1:2.5 to 1:5.

<Step 3> Preparation of IFNα-PEG-Fc Conjugate

To link the IFNα-PEG complex purified in the above step 2 to the N-terminus of an immunoglobulin Fc fragment, the immunoglobulin Fc fragment (about 53 kDa) prepared in the above step 1 was dissolved in 10 mM phosphate buffer and mixed with the IFNα-PEG complex at an IFNα-PEG complex: Fc molar ratio of 1:1, 1:2, 1:4 and 1:8. After the phosphate buffer concentration of the reaction solution was adjusted to 100 mM, a reducing agent, NaCNBH₃, was added to the reaction solution at a final concentration of 20 mM and was allowed to react at 4°C for 20 hrs with gentle agitation. Through this experiment, the optimal reaction molar ratio for IFNα-PEG complex to Fc, providing the highest reactivity and generating the fewest byproducts such as dimers, was found to be 1:2.

<Step 4> Isolation and Purification of the IFNα-PEG-Fc Conjugate

After the reaction of the above step 3, the reaction mixture was subjected to Superdex size exclusion chromatography so as to eliminate unreacted substances and byproducts and purify the IFNα-PEG-Fc protein conjugate produced. After the reaction mixture was concentrated and loaded onto a Superdex column, 10 mM phosphate buffer (pH 7.3) was passed through the column at a flow rate of 2.5 ml/min to remove unbound Fc and unreacted substances, followed by column elution to collect IFNα-PEG-Fc protein conjugate fractions. Since the collected IFNα-PEG-Fc protein conjugate fractions contained a small amount of impurities, unreacted Fc and interferon alphas dimers, cation exchange chromatography was carried out to remove the impurities. The IFNα-PEG-Fc protein conjugate fractions were loaded onto a PolyCAT LP column (PolyLC) equilibrated with 10 mM sodium acetate (pH 4.5), and the column was eluted with a linear gradient of 0.0-0.5 M NaCl in 10 mM sodium acetate buffer (pH 4.5) using 1 M NaCl. Finally, the IFNα-PEG-Fc protein conjugate was purified using an anion exchange column. The IFNα-PEG-Fc protein conjugate fractions were loaded onto a PolyWAX LP column (PolyLC) equilibrated with 10 mM Tris-Cl (pH 7.5), and the column was then eluted with a linear gradient of 0.0-0.3 M NaCl in 10 mM Tris-HCl (pH 7.5) using 1 M NaCl, thus isolating the IFNα-PEG-Fc protein conjugate in a highly pure form.

EXAMPLE 2
Preparation of IFNα-PEG-Fc Protein Conjugate

<Step 1> Preparation of Fc-PEG Complex

3.4-kDa polyethylene glycol having an aldehyde reactive group at both ends, ALD-PEG-ALD (Shearwater), was mixed with the immunoglobulin Fc fragment prepared in the step 1 of Example 1 at Fc: PEG molar ratios of 1:1, 1:2.5, 1:5, 1:10 and 1:20, wherein the Ig Fc fragment was dissolved in 100 mM phosphate buffer in an amount of 15 mg/ml. To this mixture, a reducing agent, NaCNBH₃ (Sigma), was added at a final concentration of 20 mM and was allowed to react at 4°C for 3 hrs with gentle agitation. To obtain a 1:1 complex of PEG and Fc, the reaction mixture was subjected to size exclusion chromatography using a Superdex column (Pharmacia). The Fc-PEG complex was eluted from the column using 10 mM potassium phosphate buffer (pH 6.0) as an elution buffer, and immunoglobulin Fc fragment not linked to PEG, unreacted PEG and dimer byproducts where PEG was linked to two immunoglobulin Fc fragment molecules were removed. The purified Fc-PEG complex was concentrated to about 15 mg/ml. Through this experiment, the optimal reaction molar ratio for Fc to PEG, providing the highest reactivity and generating the fewest byproducts such as dimers, was found to be 1:3 to 1:10.

<Step 2> Formation and Purification of Conjugate of the Fc-PEG Complex and Interferon Alpha

To link the Fc-PEG complex purified in the above step 1 to the N-terminus of IFNα, the Fc-PEG complex was mixed with IFNα dissolved in 10 mM phosphate buffer at Fc-PEG complex: IFNα molar ratios of 1:1, 1:1.5, 1:3 and 1:6. After the phosphate buffer, concentration of the reaction solution was adjusted to 100 mM, a reducing agent, NaCNBH₃, was added to the reaction solution at a final concentration of 20 mM and was allowed to react at 4°C for 20 hrs with gentle agitation. After the reaction was completed, unreacted substances and byproducts were removed according to the same purification method as in the step 4 of Example 1, thus isolating the Fc-PEG-IFNα protein conjugate in a highly pure form.

EXAMPLE 3
Preparation of hGH-PEG-Fc Conjugate

A hGH-PEG-Fc conjugate was prepared and purified according to the same method as in Example 1, except that drug other than interferon alphas, human growth hormone (hGH, MW: 22 kDa) was used and a hGH: PEG molar ratio was 1:5.

EXAMPLE 4
Preparation of (G-CSF)-PEG-Fc Conjugate

A (G-CSF)-PEG-Fc conjugate was prepared and purified according to the same method as in Example 1, except that drug other than interferon alphas, human granulocyte colony stimulating factor (G-CSF), was used and an G-CSF: PEG molar ratio was 1:5. On the other hand, a 14S-G-CSF-PEG-Fc protein conjugate was prepared and purified according to the same method as described above using a G-CSF derivative, 14S-G-CSF, having a serine substitution at the seventeenth amino acid residue of the native G-CSF.

EXAMPLE 5
Preparation of EPO-PEG-Fc Conjugate

An EPO-PEG-Fc conjugate was prepared and purified according to the same method as in Example 1, except that drug other than interferon alphas, human erythropoetin (EPO), was used and an EPO: PEG molar ratio was 1:5.

EXAMPLE 6
Preparation of Protein Conjugate using PEG having Different Reactive Group

An IFNα-PEG-Fc protein conjugate was prepared using PEG having a succinimidy1 propionate (SPA) reactive group at both ends, as follows. 3.4-kDa polyethylene glycol, SPA-PEG-SPA (Shearwater), was mixed with 10 mg of interferon alphas dissolved in 100 mM phosphate buffer at IFNα: PEG molar ratios of 1:1, 1:2.5, 1:5, 1:10 and 1:20. The mixture was then allowed to react at room temperature with gentle agita-
tion for 2 hrs. To obtain a 1:1 complex of PEG and interferon alpha (IFNa-PEG complex), where PEG was linked selectively to the amino group of a lysine residue of interferon alpha, the reaction mixture was subjected to Superdex size exclusion chromatography. The IFNa-PEG complex was eluted from the column using 10 mM potassium phosphate buffer (pH 6.0) as an elution buffer, and interferon alpha not linked to PEG, unreacted PEG and dimer byproducts in which two interferon alpha molecules were linked to both ends of PEG were removed. To link the IFNa-PEG complex to the amino group of a lysine residue of immunoglobulin Fe, the purified IFNa-PEG complex was concentrated to about 5 mg/mL and an IFNa-PEG-Fe conjugate was prepared and purified according to the same methods as in the steps 3 and 4 of Example 1. Through this experiment, the optimal reaction molar ratio for IFNa to PEG, providing the highest reactivity and generating the fewest byproducts such as dimers, was found to be 1:2.5 to 1:5.

On the other hand, another IFNa-PEG-Fe conjugate was prepared according to the same methods as described above using PEG having a N-hydroxysuccinimidy (NHS) reactive group at both ends, NHS-PEG-NHS (Shearwater), or PEG having a butyl aldehyde reactive group at both ends, BUA-PEG-BUA (Shearwater).

EXAMPLE 7
Preparation of Protein Conjugate using PEG having Different Molecular Weight

An IFNa-10K PEG complex was prepared using 10-kDa polyethylene glycol having an aldehyde reactive group at both ends, ALD-PEG-ALD (Shearwater). This complex was prepared and purified according to the same method as in the step 2 of Example 1. Through this experiment, the optimal reaction molar ratio for IFNa to 10-kDa PEG, providing the highest reactivity and generating the fewest byproducts such as dimers, was found to be 1:2.5 to 1:5. The purified IFNa-10K PEG complex was concentrated to about 5 mg/mL and, using this concentration, an IFNa-10K PEG-Fe conjugate was prepared and purified according to the same methods as in the steps 3 and 4 of Example 1.

EXAMPLE 8
Preparation of Fab'-S-PEG-N-Fe Conjugate (-SH Group)

<Step 1> Expression and Purification of Fab'

An E. coli transformant, BL21/poDLHb (accession number: KCCM-10511), expressing anti-tumor necrosis factor-alpha Fab', was grown in 100 mL of LB medium overnight with agitation, and was inoculated in a 5-L fermentor (Marubishi) and cultured at 30°C and 500 rpm and at an air flow rate of 20 vvm. To compensate for the insufficient nutrients for bacterial growth during fermentation, the cultures were supplemented with glucose and yeast extracts according to the fermented states of bacteria. When the cultures reached an OD600 value of 80-100, an inducer, IPTG, was added to the cultures to induce protein expression. The cultures were further cultured for 40 to 45 hrs until the OD value at 600 nm increased to 120 to 140. The fermentation fluid thus obtained was centrifuged at 20,000 x g for 30 min. The supernatant was collected, and the pellet was discarded.

The supernatant was subjected to the following three-step column chromatography to purify anti-tumor necrosis factor-alpha Fab'. The supernatant was loaded onto a HiTrap protein G column (5 ml, Pharmacia) equilibrated with 20 mM phosphate buffer (pH 7.0), and the column was eluted with 100 mM glycine (pH 3.0). The collected Fab' fractions were then loaded onto a Superdex 200 column (Pharmacia) equilibrated with 10 mM sodium phosphate buffer (PBS, pH 7.3), and this column was eluted with the same buffer. Finally, the second Fab' fractions were loaded onto a polyCAT 21×250 column (PolyLC), and this column was eluted with a linear NaCl gradient of 0.15-0.4 M in 10 mM acetate buffer (pH 4.5), thus providing highly pure anti-tumor necrosis factor-alpha Fab' fractions.

<Step 2> Preparation and Purification of Fe-PEG Complex

To link a PEG linker to the N-terminus of an immunoglobulin Fe, the immunoglobulin Fe prepared according to the same method as in the step 1 of Example 1 was dissolved in 100 mM phosphate buffer (pH 6.0) at a concentration of 5 mg/mL and, was mixed with NHS-PEG-MAL (3.4 kDa, Shearwater) at an Fe: PEG molar ratio of 1:10, followed by incubation at 4°C for 12 hrs with gentle agitation.

After the reaction was completed, the reaction buffer was exchanged with 20 mM sodium phosphate buffer (pH 6.0) to remove unbound NHS-PEG-MAL. Then, the reaction mixture was loaded onto a polyCAT column (PolyLC). The column was eluted with a linear NaCl gradient of 0.15-0.5 M in 20 mM sodium phosphate buffer (pH 6.0). During this elution, the immunoglobulin Fe-PEG complex was eluted earlier than unreacted immunoglobulin Fe, and the unreacted Ig Fe was eluted later, thereby eliminating the unreacted Ig Fe molecules.

<Step 3> Preparation and Purification of Fab'-S-PEG-N-Fe Conjugate (-SH Group)

To link the immunoglobulin Fe-PEG complex to a cysteine group of the Fab', the Fab' purified in the above step 1 was dissolved in 100 mM sodium phosphate buffer (pH 7.3) at a concentration of 2 mg/mL and, was mixed with the immunoglobulin Fe-PEG complex prepared in the above step 2 at a Fab': complex molar ratio of 1:5. The reaction mixture was concentrated to a final protein concentration of 50 mg/mL and incubated at 4°C for 24 hrs with gentle agitation.

After the reaction was completed, the reaction mixture was loaded onto a Superdex 200 column (Pharmacia) equilibrated with 10 mM sodium phosphate buffer (pH 7.3), and the column was eluted with the same buffer at a flow rate of 1 ml/min. The coupled Fab'-S-PEG-N-Fe conjugate was eluted relatively earlier due to its high molecular weight, and unreacted immunoglobulin Fe-PEG complex and Fab' were eluted later, thereby eliminating the unreacted molecules. To completely eliminate unreacted immunoglobulin Fe-PEG, the collected Fab'-S-PEG-N-Fe conjugate fractions were again loaded onto a polyCAT 21×250 column (PolyLC), and this column was eluted with a linear NaCl gradient of 0.15-0.5 M in 20 mM sodium phosphate buffer (pH 6.0), thus providing a pure Fab'-S-PEG-N-Fe conjugate comprising the Fe-PEG complex linked to an -SH group near the C-terminus of the Fab'.

EXAMPLE 9
Preparation of Fab'-N-PEG-N-Fe conjugate (N-terminus)

<Step 1> Preparation and Purification of Fab'-PEG Complex (N-terminus)

40 mg of the Fab' purified in the step 1 of Example 8 was dissolved in 100 mM sodium phosphate buffer (pH 6.0) at a
concentration of 5 mg/ml, and was mixed with butyl ALD-PEG-butyl ALD (3.4 kDa, Nektar) at a Fab': PEG molar ratio of 1:5. A reducing agent, NaCNBH₃, was added to the reaction mixture at a final concentration of 20 mM, and the reaction mixture was then allowed to react at 4°C for 2 hrs with gentle agitation.

After the reaction was completed, the reaction buffer was exchanged with 20 mM sodium phosphate buffer (pH 6.0). Then, the reaction mixture was loaded onto a polyCAT column (PolyLC). The column was eluted with a linear NaCl gradient of 0.15-0.4 M in 20 mM acetate buffer (pH 4.5). During this column elution, the Fab'-PEG complex comprising the PEG linker lined to the N-terminus of the Fab' was eluted earlier than unreacted Fab', and the unreacted Fab' was eluted later, thereby eliminating the unreacted Fab' molecules.

**Step 2: Preparation and Purification of Fab'-N-PEG-N-Fc Conjugate**

To link the Fab'-PEG complex purified in the above step 1 to the N-terminus of an immunoglobulin Fc, the Fab'-PEG complex was dissolved in 100 mM sodium phosphate buffer (pH 6.0) at a concentration of 10 mg/ml, and was mixed with the immunoglobulin Fc dissolved in the same buffer at a Fab'-PEG complex: Fc molar ratio of 1:5. After the reaction mixture was concentrated to a final protein concentration of 50 mg/ml, a reducing agent, NaCNBH₃, was added to the reaction mixture at a final concentration of 20 mM, and the reaction mixture was then reacted at 4°C for 24 hrs with gentle agitation.

After the reaction was completed, the reaction mixture was loaded onto a Superdex 200 column (Pharmacia) equilibrated with 10 mM sodium phosphate buffer (pH 7.3), and the column was eluted with the same buffer at a flow rate of 1 ml/min. The coupled Fab'-N-PEG-N-Fc conjugate was eluted relatively earlier due to its high molecular weight, and unreacted immunoglobulin Fc and Fab'-PEG complex were eluted later, thereby eliminating the unreacted molecules. To completely eliminate the unreacted immunoglobulin Fc molecules, the collected Fab'-N-PEG-N-Fc conjugate fractions were again loaded onto a polyCAT 21 x 250 column (PolyLC), and this column was eluted with a linear NaCl gradient of 0.15-0.5 M in 20 mM sodium phosphate buffer (pH 6.0), thus providing a pure Fab'-N-PEG-N-Fc conjugate comprising the immunoglobulin Fc-PEG complex linked to the N-terminus of the Fab'.

**EXAMPLE 10**

**Preparation and Purification of Deglycosylated Immunoglobulin Fc**

200 mg of an immunoglobulin Fc prepared according to the same method as in Example 1 was dissolved in 100 mM phosphate buffer (pH 7.5) at a concentration of 2 mg/ml, and was mixed with 300 U/mg of a deglycosylase, PNGase F (NEB). The reaction mixture was allowed to react at 37°C for 24 hrs with gentle agitation. Then, to purify the deglycosylated immunoglobulin Fc, the reaction mixture was loaded onto a SP Sepharose FF column (Pharmacia), and the column was eluted with a linear NaCl gradient of 0.1-0.6 M in 10 mM acetate buffer (pH 4.5) using 1 M NaCl. The native immunoglobulin Fc was eluted earlier, and the deglycosylated immunoglobulin Fc (DG Fc) was eluted later.
of synthesized primers represented by SEQ ID Nos. 2 and 3 were used. SEQ ID NO. 1 is a nucleotide sequence starting from
the 10th residue, serine, of 12 amino acid residues, below, of the hinge region of IgG4. SEQ ID NO. 3 was
designed to have a nucleotide sequence encoding a Cys2 domain having alamine as a first amino acid residue. SEQ ID NO. 2 was designed to have a BamHI recognition site containing a stop codon.

To clone each of the amplified IgG4 constant region fragments into an expression vector containing an E. coli secretory sequence derivative, the pT14S1SH-4T20V22Q (Korean Pat. No. 38061) developed prior to the present invention was used. This expression vector contains a heat-stable enterotoxin secretory sequence derivative that has a nucleotide sequence represented by SEQ ID NO. 4. To facilitate cloning, a Stul recognition site was inserted into an end of the E. coli heat-stable enterotoxin secretory sequence derivative of the pT14S1SH-4T20V22Q plasmid through site-directed mutagenesis using a pair of primers represented by SEQ ID Nos. 5 and 6 to induce mutagenesis to introduce the Stul site at a nucleotide sequence coding for the last amino acid residue of the secretory sequence. This insertion of the Stul site was found to be successful by DNA sequencing. The resulting pT14S1SH-4T20V22Q plasmid containing a Stul site was designated as pmSTH. The pmSTH plasmid was treated with Stul and BamHI and subjected to agarose gel electrophoresis, and a large fragment (4.7 kb), which contains the E. coli heat-stable enterotoxin secretory sequence derivative, was purified. Then, the amplified gene fragments were digested with BamHI and ligated with the linearized expression vector, thus providing pSTHDC4Fc and pSTHDC4Gm.

The final expression vectors were individually transformed into E. coli BL21(DE3), and the resulting transformants were designated as BL21/pSTHDC4Fc (HM10932) and BL21/
pSTHDC4Gm (HM10933), which were deposited at the Korean Culture Center of Microorganisms (KCCM) on Sep. 15, 2004 and assigned accession numbers KCCM-10597 and
KCCM-10598, respectively. Thereafter, when the cultures reached an OD_{600} value of 80, an inducer, IPTG, was added to the cultures to induce protein expression. The cultures were further cultured for 40 to 45 hrs until the OD value at 600 nm increased to 100 to 120. The E. coli cells collected from the fermentation fluids were disrupted, and the resulting cell lysates were subjected to two-step column chromatography to purify the recombinant immunoglobulin constant region derivatives present in the cytosol of E. coli. 5 ml of a protein-A affinity column (Pharmacia) was equilibrated with PBS, and the cell lysates were loaded onto the column at a flow rate of 5 ml/min. Unbound proteins were washed out with PBS, and bound proteins were eluted with 100 mM citrate (pH 3.0). The collected fractions were desalted using a HiPrep 26/10 desalting column (Pharmacia) with 10 mM Tris buffer (pH 8.0). Then, secondary union exchange column chromatography was carried out using 50 ml of a Q HP 26/10 column (Pharmacia). The primary purified recombinant aglycosylated immunoglobulin Fc fractions were loaded onto the Q-Sepharose HP 26/10 column, and the column was eluted with a linear gradient of 0-0.2 M NaCl in 10 mM Tris buffer (pH 8.0), thus providing a highly pure recombinant aglycosylated immunoglobulin Fc (AG Fc) derivative, IgG4 delta-Cys and a highly pure IgG4 monomer fraction.

EXEMPLARY EXAMPLE 13
Preparation of Conjugate of IFNα-PEG Complex
and Recombinant AG Fc Derivative

According to the same methods as in Examples 1 and 11, the IFNα-PEG complex was linked to the N terminus of the IgG4 delta-Cys as an AG Fc derivative prepared in Example 12. After the coupling reaction, unreacted substances and byproducts were removed from the reaction mixture, and the thus-produced IFNα-PEG-AG Fc protein conjugate (I) was primarily purified using 50 ml of a Q HP 26/10 column (Pharmacia) and further purified by a high-pressure liquid chromatographic assay using a polycAT 21.5x250 column (polYLC), thus purifying the conjugate to a high degree. The coupling reaction solution was desalted using a HiPrep 26/10 desalting column (Pharmacia) with 10 mM Tris buffer (pH 8.0). Then, the reaction solution was then loaded onto 50 ml of a Q HP 26/10 column (Pharmacia) at a flow rate of 8 ml/min, and this column was eluted with a linear NaCl gradient of 0-0.2 M to obtain desired fractions. The collected fractions were then loaded onto a polycAT 21.5x250 column equilibrated with 10 mM acetate buffer (pH 5.2) at a flow rate of 15 ml/min, and this column was eluted with a linear NaCl gradient of 0.1-0.3 M, thus providing highly pure fractions. According to the same method described above, another IFNα-PEG-AG Fc protein conjugate (II) was prepared using another AG Fc derivative prepared in Example 12, IgG4 monomer.

EXEMPLARY EXAMPLE 14
Preparation of EPO-PEG-recombinant AG Fc Derivative Conjugate

According to the same method as in Example 13, an EPO-PEG-recombinant AG Fc derivative conjugate was prepared by linking an AG Fc derivative, IgG4 delta-Cys, to the EPO-PEG complex.

COMPARATIVE EXAMPLE 1
Preparation of IFNα-40K PEG Complex

5 mg of interferon alpha was dissolved in 100 mM phosphate buffer to obtain a final volume of 5 ml, and was mixed with 40-kDa activated methoxy-PEG-aldehyde (Sherwood), at an IFNα-40-kDa PEG molar ratio of 1:4. To this mixture, a reducing agent, NaCNBH3, was added at a final concentration of 20 mM and was allowed to react at 4°C for 18 hrs with gentle agitation. To inactivate PEG, which did not react with IFNα, Ethanalamine was added to the reaction mixture at a final concentration of 50 mM. A Sephadex G-25 column (Pharmacia) was used to remove unreaceted PEG and exchange the buffer with another buffer. First, this column was equilibrated with two column volumes (CV) of 10 mM Tris-HCl buffer (pH 7.5), and was loaded with the reaction mixture. Flow throughs were detected by measuring the absorbance at 260 nm using a UV spectrophotometer. When the column was eluted with the same buffer, interferon alpha modified by adding PEG having a higher molecular weight to its N-terminus was eluted earlier, and unreaceted PEG was eluted later, thus allowing isolation of only IFNα-40K PEG.

The following chromatography was carried out to further purify the IFNα-40K PEG complex from the collected frac-
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Preparation of IFNa-PEG-albumin Conjugate

To link the IFNa-PEG complex purified in the step 2 of Example 1 to the N-terminus of albumin, the IFNa-PEG complex was mixed with human serum albumin (HSA, about 67 kDa, Green Cross) dissolved in 10 mM phosphate buffer at an IFNa-PEG complex:albumin molar ratio of 1:1, 1:2, 1:4 and 1:8. After the phosphate buffer concentration of the reaction solution was adjusted to 100 mM, a reducing agent, NaCNBH₃, was added to the reaction solution at a final concentration of 20 mM and was allowed to react at 4°C for 20 hrs with gentle agitation. Through this experiment, the optimal reaction molar ratio for IFNa-PEG complex to albumin, providing the highest reactivity and generating the fewest byproducts such as dimers, was found to be 1:2.

After the coupling reaction, the reaction mixture was subjected to size exclusion chromatography using a Superdex²⁵⁰ column (Pharmacia) so as to eliminate unreacted substances and byproducts and purify the IFNa-PEG-albumin protein conjugate produced. After the reaction mixture was concentrated and loaded onto the column, 10 mM sodium acetate buffer passed through the column at a flow rate of 2.5 mL/min to remove unbound albumin and unreacted substances, followed by column elution to purify only IFNa-PEG-albumin protein conjugate. Since the collected IFNa-PEG-albumin protein conjugate fractions contained a small amount of impurities, unreacted albumin and interferon alpha dimers, cation exchange chromatography was carried out to remove the impurities. The IFNa-PEG-albumin protein conjugate fractions were loaded onto a SP5PW column (Waters) equilibrated with 10 mM sodium acetate (pH 4.5), and the column was eluted with a linear gradient of 0-4.5 M NaCl in 10 mM sodium acetate buffer (pH 4.5) using 1 M NaCl, thus isolating the IFNa-PEG-albumin protein conjugate in a highly pure form.

According to the same method as described above, albumin was conjugated to human growth hormone, G-CSF, and a derivative of G-CSF, thus providing hGH-PEG-albumin, G-CSF-PEG-albumin and ¹²⁵S-G-CSF-PEG-albumin conjugates.

COMPARATIVE EXAMPLE 2

Preparation of Fab'-S-40K PEG Complex

The free cysteine residue of the Fab' purified in the step 1 of Example 8 was activated by incubation in an activation buffer (20 mM sodium acetate (pH 4.0), 0.2 mM DTT) for 1 hr. After the buffer was exchanged by a PEG modification buffer, 50 mM potassium phosphate (pH 6.5), maleimide-PEG (MW: 40 kDa, Shearwater) was added thereto at a Fab'-40-kDa PEG molar ratio of 1:10 and was reacted to react at 4°C for 24 hrs with gentle agitation.

After the reaction was completed, the reaction solution was loaded onto a Superdex 200 column (Pharmacia) equilibrated with 10 mM sodium phosphate buffer (pH 7.3), and the column was eluted with the same buffer at a flow rate of 1 ml/min. The Fab' conjugated 40-kDa PEG (Fab'-40K PEG) was eluted relatively earlier due to its high molecular weight, and unreacted Fab' was eluted later, thereby eliminating the unreacted Fab'. To completely eliminate the unreacted Fab', the collected Fab'-40K PEG complex fractions were again loaded onto a polyCat 21×250 column (PolyLC), and this column was eluted with a linear NaCl gradient of 0.15-0.5 M in 20 mM sodium phosphate buffer (pH 4.5), thus providing a pure Fab'-S-40K PEG complex comprising 40-kDa PEG linked to an -SH group of the Fab'.

COMPARATIVE EXAMPLE 3

Preparation of Fab'-S-40K PEG Complex

The free cysteine residue of the Fab' purified in the step 1 of Example 8 was activated by incubation in an activation buffer (20 mM sodium acetate (pH 4.0), 0.2 mM DTT) for 1 hr. After the buffer was exchanged by a PEG modification buffer, 50 mM potassium phosphate (pH 6.5), maleimide-PEG (MW: 40 kDa, Shearwater) was added thereto at a Fab'-40-kDa PEG molar ratio of 1:10 and was reacted to react at 4°C for 24 hrs with gentle agitation.

After the reaction was completed, the reaction solution was loaded onto a Superdex 200 column (Pharmacia) equilibrated with 10 mM sodium phosphate buffer (pH 7.3), and the column was eluted with the same buffer at a flow rate of 1 ml/min. The Fab' conjugated 40-kDa PEG (Fab'-40K PEG) was eluted relatively earlier due to its high molecular weight, and unreacted Fab' was eluted later, thereby eliminating the unreacted Fab'. To completely eliminate the unreacted Fab', the collected Fab'-40K PEG complex fractions were again loaded onto a polyCat 21×250 column (PolyLC), and this column was eluted with a linear NaCl gradient of 0.15-0.5 M in 20 mM sodium phosphate buffer (pH 4.5), thus providing a pure Fab'-S-40K PEG complex comprising 40-kDa PEG linked to an -SH group of the Fab'.
shows the result of size exclusion chromatography of the purified IFNα-PEG-Fc conjugate, wherein a single peak was observed. This result indicates that the purified protein conjugate does not contain multimeric impurities such as a dimer, a trimer or a higher number of monomers.

When a physiologically active polypeptide conjugated to Fc was quantitatively analyzed using an antibody specific to the physiologically active polypeptide, the antibody was prevented from binding to the polypeptide, resulting in a value lower than an actual value calculated by the chromatography. In the case of the IFNα-PEG-Fc conjugate, an ELISA resulted in an ELISA value corresponding to about 30% of an actual value.

1-3: Evaluation of Purity and Mass of the Protein Conjugates

The protein conjugates prepared in the above Examples were subjected to size exclusion chromatography, and absorbance was measured at 280 nm. As a result, the IFNα-PEG-Fc, hGH-PEG-Fc, G-CSF-PEG-Fc and 17S-ser-G-CSF-PEG-Fc conjugates displayed a single peak at the retention time of a 70 to 80-kDa substance.

On the other hand, reverse phase HPLC was carried out to determine purities of the protein conjugates prepared in Examples 1, 11 and 13, IFNα-PEG-Fc, IFNα-PEG-DG Fc and IFNα-PEG-recombinant AG Fc derivative. A reverse phase column (259 VHP54 column, Vydac) was used. The column was eluted with a 40-100% acetonitrile gradient with 0.5% TFA, and purities were analyzed by measuring absorbance at 280 nm. As a result, as shown in FIG. 8, the samples contain no unbound interferon or immunoglobulin Fc, and all of the protein conjugates, IFNα-PEG-Fc (A), IFNα-PEG-DG Fc (B) and IFNα-PEG-recombinant AG Fc derivative (C), were found to have purity greater than 96%.

To determine accurate molecular weights of the purified protein conjugates, mass for each conjugate was analyzed using a high-throughput MALDI-TOF mass spectrometer (Voyager DE-STR, Applied Biosystems). Sinapinic acid was used as a matrix. 0.5 μl of each test sample was coated onto a sample slide and air-dried, again mixed with the equal volume of a matrix solution and air-dried, and introduced into an ion source. Detection was carried out in a positive fashion using a linear mode TOF analyzer. Ions were accelerated with a split extraction source operated with delayed extraction (DE) using a delayed extraction time of 750 nsec to 1500 nsec at a total acceleration voltage of about 2.5 kV.

The molecular weights observed by MALDI-TOF mass spectrometry for the Fc protein conjugates prepared in Examples are given in Table 1, below. FIG. 5 shows the result of MALDI-TOF mass spectrometry of the EPO-PEG-Fc conjugate, and FIG. 7 shows the results of MALDI-TOF mass spectrometry of the IFNα-PEG-Fc and IFNα-PEG-DG Fc conjugates. As a result, the EPO-PEG-Fc protein conjugate was found to have a purity of more than 95% and a molecular weight very close to a theoretical MW. Also, EPO was found to couple to the immunoglobulin Fc fragment at a ratio of 1:1.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Theoretical MW (kDa)</th>
<th>Measured MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNα-PEG-Fc (E. 1)</td>
<td>75.4</td>
<td>75.9</td>
</tr>
<tr>
<td>hGH-PEG-Fc (E. 5)</td>
<td>78.4</td>
<td>78.6</td>
</tr>
<tr>
<td>G-CSF-PEG-Fc (E. 4)</td>
<td>75.3</td>
<td>75.9</td>
</tr>
<tr>
<td>17S-G-CSF derivative-PEG-Fc (E. 4)</td>
<td>75.0</td>
<td>75.9</td>
</tr>
<tr>
<td>EPO-PEG-Fc (E. 5)</td>
<td>91.4</td>
<td>91.0</td>
</tr>
</tbody>
</table>

In addition, when the Fc and DG Fc prepared in Example 10 were examined for their molecular weights by MALDI-TOF mass spectrometry, the DG Fc was found to be 50 kDa, which is about 3-kDa less than native Fc (FIG. 6a). Since the 3-kDa MW corresponds to the theoretical size of sugar moieties, the results demonstrate that the sugar moieties are completely removed.

Table 2, below, shows the results of MALDI-TOF mass spectrometry of the IFNα-PEG-DG Fc conjugate prepared in Example 11 and the IFNα-PEG-recombinant AG Fc derivative conjugates (I and II) prepared in Example 13. The IFNα-PEG-DG Fc conjugate was found to be 3 kDa lighter, and the IFNα-PEG-recombinant AG Fc derivative conjugate (I) to be about 3-4 kDa lighter, than the IFNα-PEG-Fc conjugate of 75.9 kDa. The IFNα-PEG-recombinant AG Fc derivative conjugate (II) coupled to an Fc monomer showed a molecular weight decreased by 24.5 kDa corresponding to the molecular weight of the Fc monomer.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Theoretical MW (kDa)</th>
<th>Measured MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNα-PEG-DG Fc (E. 11)</td>
<td>72.8</td>
<td>73.0</td>
</tr>
<tr>
<td>IFNα-PEG-recombinant AG Fc derivative (I) (E. 13)</td>
<td>72.3</td>
<td>72.2</td>
</tr>
<tr>
<td>IFNα-PEG-recombinant AG Fc derivative (II) (E. 13)</td>
<td>46.8</td>
<td>46.6</td>
</tr>
</tbody>
</table>

EXPERIMENTAL EXAMPLE 2

Pharmacokinetic Analysis

Native forms of physiologically active proteins (controls) and the protein conjugates prepared in Examples and Comparative Examples, -40K PEG complexes, -PEG-albumin conjugates, -PEG-Fc conjugates, -PEG-DG Fc conjugates and -PEG-recombinant AG Fc derivative conjugates, were evaluated for serum stability and pharmacokinetic parameters in SD rats (five rats per group). The controls, and the -40K PEG complexes, -PEG-albumin conjugates, -PEG-Fc conjugates, -PEG-DG Fc conjugates and -PEG-recombinant AG Fc derivative conjugates (test groups) were individually injected subcutaneously at a dose of 100 μg/kg. After the subcutaneous injection, blood samples were collected at 0.5, 1, 2, 4, 6, 12, 24, 30, 48, 72 and 96 hrs in the control groups, and, in the test groups, at 1, 6, 12, 24, 30, 48, 72, 96, 120, 240 and 288 hrs. The blood samples were collected in tubes with an anticoagulant, heparin, and centrifuged for 5 min using an Eppendorf high-speed micro centrifuge to remove blood cells. Serum protein levels were measured by ELISA using antibodies specific to the physiologically active proteins.

The results of pharmacokinetic analyses of the native forms of IFNα, hGH, G-CSF and EPO, and -40K PEG complexes thereof, -PEG-albumin conjugates thereof, -PEG-Fc conjugates thereof and -PEG-DG Fc conjugates thereof, are given in Tables 3 to 7, below. In the following tables, T1/2 indicates the time taken to reach the maximal drug serum concentration, Tmax indicates the serum half-life of a drug, and MRT (mean residence time) indicates the mean time that a drug molecule resides in the body.
TABLE 3

Pharmacokinetics of interferon alpha

<table>
<thead>
<tr>
<th>IFNα</th>
<th>IFNα-PEG-albumin</th>
<th>IFNα-PEG-Fc</th>
<th>IFNα-PEG-DG Fc</th>
<th>IFNα-PEG-DG Fc derivative</th>
<th>IFNα-PEG-DG Fc derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>IFNα-40K PEG</td>
<td>IFNα-40K PEG</td>
<td>IFNα-40K PEG</td>
<td>IFNα-40K PEG</td>
<td>IFNα-40K PEG</td>
</tr>
<tr>
<td>Tmax (hr)</td>
<td>1.0</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>T1/2 (hr)</td>
<td>1.7</td>
<td>5.9</td>
<td>5.9</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>MRT (hr)</td>
<td>2.1</td>
<td>13.0</td>
<td>13.0</td>
<td>13.0</td>
<td>13.0</td>
</tr>
</tbody>
</table>

Comparative Example 1. Also, the IFNα-PEG-Fc protein conjugate of the present invention was found to be superior in serum half-life to IFNα-PEG-albumin, which has a half-life of 17.1 hrs.

On the other hand, as shown in Table 3 and FIG. 11, the IFNα-PEG-DG Fc conjugate had a serum half-life of 71.0 hrs, which was almost the same as the IFNα-PEG-Fc conjugate, indicating that the deglycosylation of Fc does not greatly affect the in vivo stability of the IFNα-PEG-DG Fc conjugate. Also, the conjugate prepared using the recombinant AG Fc derivative produced by a recombinant method was found to have an effect identical to that of the native form-derived DG Fc. However, the serum half-life of a complex coupled to an Fc monomer was about half that of a complex coupled to a normal Fc dimer.

As shown in Table 4, human growth hormone also showed an extended serum half-life when conjugated to the IgG Fc fragment according to the present invention. That is, compared to the native form (1.1 hrs), the hGH-40K PEG complex and hGH-PEG-albumin conjugate had slightly increased half-lives of 7.7 hrs and 5.9 hrs, respectively, whereas the hGH-PEG-Fc protein conjugate of the present invention displayed a greatly extended serum half-life of 11.8 hrs.

As apparent from the pharmacokinetic data of G-CSF and its derivative in Table 5 and 6, the G-CSF-PEG-Fc and 15S-G-CSF-PEG-Fc conjugates displayed a much longer serum half-life than the 40K PEG complex and -PEG-albumin conjugate. The immunoglobulin Fc fragment was found in the serum to prolong the duration of action of physiologically active proteins in native forms, as well as in their derivatives having alterations of certain amino acid residues in similar levels to the native forms. From these results, it is easily predictable that the method of the present invention will have a similar effect on other proteins and their derivatives.

As shown in Table 7 and FIG. 10, the conjugation of the native glycosylated EPO to the Fc fragment also resulted in an increase in serum half-life. That is, EPO had a serum half-life of 9.4 hrs in the native form, and a prolonged serum half-life of 18.4 hrs when highly glycosylated to improve serum stability. The EPO-PEG-Fc conjugate, comprising EPO coupled to the immunoglobulin Fc fragment according to the present invention, displayed a markedly prolonged serum half-life of 61.5 hrs. Also, when conjugated to the E. coli-derived recombinant glycosylated (AG) Fc derivative, the half-life of EPO increased to 87.9 hrs, indicating that the aglycosylation of the Fc fragment allows the preparation of a protein conjugate not affecting serum stability of the protein without antibody functions.

As apparent from the above results, the protein conjugates covalent-bonded to the immunoglobulin Fc fragment through
a non-peptide polymer according to the present invention displayed serum half-lives increased several to several tens to that of the native form. Also, when the immunoglobulin Fc was aglycosylated by production in E. coli or deglycosylated by enzyme treatment, its effect of increasing the serum half-life of its protein conjugate was maintained at a similar level.

In particular, compared to proteins modified with 40-kDa PEG having the longest duration of action among PEG molecules for increasing the duration of action of proteins in the serum, the immunoglobulin Fc protein conjugates had much higher superior serum stability. In addition, compared to protein conjugates coupled to albumin instead of the immunoglobulin Fc, the protein conjugates of the present invention displayed excellent serum stability, indicating that the protein conjugates of the present invention are effective in developing long-acting forms of protein drugs. These results, that the present protein conjugates have excellent effects on serum stability and MRT in a broad range of proteins including colony stimulating factor derivatives by point mutation compared to conventional PEG- or albumin-conjugated proteins, indicate that the stability and duration-extending effects of the present protein conjugates are applicable to other physiologically active polypeptides.

On the other hand, when the IFNα-10K PEG-Fc protein conjugate (Example 7) prepared using a non-peptide polymer, 10-kDa PEG, was evaluated for its serum half-life according to the same method as described above, it showed a serum half-life of 48.8 hrs, which was somewhat shorter than the serum half-life (79.7 hrs) of a protein conjugate prepared using 3.4-kDa PEG.

In addition, the serum half-lives of the protein conjugates decrease with increasing molecular weight of the non-peptide polymer PEG. These results indicate that the major factor increasing the serum stability and duration of the protein conjugates is the conjugated immunoglobulin Fc fragment rather than the non-peptide polymer.

Even when the reactive group of PEG was exchanged with a reactive group other than the aldehyde group, protein conjugates with the PEG showed similar patterns in apparent molecular weight and serum half-life to those coupled to PEG having an aldehyde reactive group.

**EXPERIMENTAL EXAMPLE 3**

**Pharmacokinetic Analysis II**

To determine the serum half-lives of the Fab’-S-PEG-N-Fc and Fab’-N-PEG-N-Fc conjugates prepared in Example 8 and 9 and the Fab’-S-40K PEG complex prepared in Comparative Example 3, drug pharmacokinetic analysis was carried out according to the same method as in Experimental Example 2 using Fab’ as a control, the conjugates and the complex. The results are given in Fig. 12.

As shown in Fig. 12, the Fab’-S-PEG-N-Fc and Fab’-N-PEG-N-Fc conjugates displayed a serum half-life prolonged two or three times compared to the Fab’ or Fab’-S-40K PEG complex.

**EXPERIMENTAL EXAMPLE 4**

**Evaluation of Intracellular Activity of the Protein Conjugates**

Comparison of the IFNα Protein Conjugates for Intracellular Activity

To compare the intracellular activity of the IFNα protein conjugates, the IFNα-PEG-Fc (Example 1), IFNα-PEG-DG

<table>
<thead>
<tr>
<th>Table 8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conc. (ng/ml)</strong></td>
</tr>
<tr>
<td>Native IFNα</td>
</tr>
<tr>
<td>IFNα-40K PEG</td>
</tr>
<tr>
<td>IFNα-PEG-albumin</td>
</tr>
<tr>
<td>IFNα-PEG-Fc</td>
</tr>
<tr>
<td>IFNα-PEG-DG Fc</td>
</tr>
<tr>
<td>IFNα-PEG-recombinant AG Fc derivative</td>
</tr>
</tbody>
</table>

As shown in Table 8, the IFNα-40K PEG decreased in activity to 4.8% of the native IFNα. Especially, as the size of the PEG moieties increased, a protein conjugate has improved serum stability but gradually decreased activity. Interferon alpha was reported to have in vitro activities of 25% when modified with 12-kDa PEG and about 7% when modified with 40-kDa PEG (P. Bailon et al., *Bioconjugate Chem.*, 12:195-202, 2001). That is, since a protein conjugate has a longer half-life but sharply decreases in biological activity as the molecular weight of PEG moieties increase, there is a need for the development of a protein conjugate having a longer serum half-life and a stronger activity. In addition, the IFNα-PEG-albumin conjugate displayed a weak activity of about 5.2% compared to the native IFNα. In contrast, the IFNα-PEG-Fc and IFNα-PEG-DG Fc conjugates of the present invention exhibited a markedly improved relative activity of 28.1% and 25.7% compared to the native IFNα. Also, the conjugation of IFNα to the recombinant AG Fc derivative resulted in a similar increase in activity. From these
As shown in Table 9, also in the case of human growth hormone, the conjugation to 40-kDa PEG (hGH-PEG) resulted in a decrease in activity to about 7.6% of the native form, and the hGH-PEG-albumin conjugate displayed a low in vitro activity that was about 5.2% of the native hGH. However, the hGH-PEG-Fc conjugate of the present invention markedly increased in relative activity to greater than 28% compared to the native hGH. From these results, it is expected that human growth hormone linked to the immunoglobulin Fc fragment has a markedly increased serum half-life and a greatly improved in vivo pharmaceutical efficacy. In addition, it is believed that the increased activity of the immunoglobulin Fc protein conjugates of the present invention is due to the increased serum stability and preserved binding affinity to receptors due to the immunoglobulin Fc or due to the space formed by the non-peptide polymer. These effects are predicted to be applicable to immunoglobulin Fc protein conjugates coupled to other pharmaceutically active proteins.

### TABLE 9

<table>
<thead>
<tr>
<th>Conc. (ng/ml)</th>
<th>Specific activity* (U/mg)</th>
<th>Relative activity (%) for native hGH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native hGH</td>
<td>2.72E+06</td>
<td>100</td>
</tr>
<tr>
<td>hGH (standard available from NIHSEC)</td>
<td>2.38E+06</td>
<td>95.2</td>
</tr>
<tr>
<td>hGH-PEG</td>
<td>100</td>
<td>7.6</td>
</tr>
<tr>
<td>hGH-PEG-albumin</td>
<td>100</td>
<td>5.2</td>
</tr>
<tr>
<td>hGH-PEG-Fc</td>
<td>100</td>
<td>28.1</td>
</tr>
</tbody>
</table>

Specific activity* = 1/ED50 x 10^6 (ED50 protein amount required for 50% of maximum cell growth)

As shown in Table 9, also in the case of human growth hormone, the conjugation to 40-kDa PEG (hGH-PEG) resulted in a decrease in activity to about 7.6% of the native form, and the hGH-PEG-albumin conjugate displayed a low in vitro activity that was about 5.2% of the native hGH. However, the hGH-PEG-Fc conjugate of the present invention markedly increased in relative activity to greater than 28% compared to the native hGH. From these results, it is expected that human growth hormone linked to the immunoglobulin Fc fragment has a markedly increased serum half-life and a greatly improved in vivo pharmaceutical efficacy. In addition, it is believed that the increased activity of the immunoglobulin Fc protein conjugates of the present invention is due to the increased serum stability and preserved binding affinity to receptors due to the immunoglobulin Fc or due to the space formed by the non-peptide polymer. These effects are predicted to be applicable to immunoglobulin Fc protein conjugates coupled to other pharmaceutically active proteins.
Cytotoxicity Neutralization Assay for the Fab' Conjugates

An in vitro activity assay was carried out using the Fab'-S-PEG-N-Fc and Fab'-N-PEG-N-Fc conjugates prepared in Example 8 and 9 and the Fab'-S-40K PEG complex prepared in Comparative Example 3. Through a cytotoxicity assay based on measuring TNFα-mediated cytolysis, the Fab' conjugates were evaluated to determine whether they neutralize TNFα-induced apoptosis in a mouse fibroblast cell line, L929 (ATCC CRL-2148).

The Fab'-S-PEG-N-Fc and Fab'-N-PEG-N-Fc conjugate and the Fab'-S-40K PEG complex were serially two-fold diluted, and 100-μl aliquots were placed onto wells of a 96-well plate. rTNFα (R&D systems) and actinomycin D (Sigma) used as a RNA synthesis inhibitor were added to each well at final concentrations of 10 ng/ml and 1 μg/ml, respectively, incubated for 30 min in an incubator at 37°C, with 5% CO2, and transferred to a microplate for assay. L929 cells were added to each well at a density of 5x10^3 cells/50 μl medium and cultured for 24 hrs in an incubator at 37°C, with 5% CO2. After the culture medium was removed, 50 μl of MTT (Sigma) dissolved in PBS at a concentration of 5 mg/ml was added to each well, and the cells were further cultured for about 4 hrs in an incubator at 37°C, with 5% CO2, 150 μl of DMSO was added to each well, and the degree of cytotoxicity neutralization was determined by measuring the absorbance at 540 nm. As a control, the Fab' purified in the step 1 of Example 8 was used.

As shown in FIG. 13, all of the protein conjugates used in this test had a similar titer to the Fab'. These results indicate that, when a protein conjugate is prepared, this will lead an immunoglobulin Fc to a free cysteine residue near the N-terminus or C-terminus of a Fab' through PEG, the Fab' exhibits a markedly increased serum half-life and a high in vivo activity.

Complement-dependent Cytotoxicity (CDC) Assay

To determine whether the derivatives prepared in Examples and proteins corresponding to the constant regions of immunoglobulins, expressed in the E. coli transformants and purified, bind to human C1q, an enzyme linked immunosorbent assay (ELISA) was carried out as follows. As test groups, immunoglobulin constant regions produced by the HM10932 and HM10927 transformants, deposited at the Korean Culture Center of Microorganisms (KCCM) on Sep. 15, 2004 and assigned accession numbers KCCM-10597, KCCM-10598, and the derivatives prepared in the above Examples were used. As standards, a glycosylated immunoglobulin (IVIG-globulin S, Green Cross PBM) and several commercially available antibodies used as therapeutic antibodies were used. The test and standard samples were prepared in 10 mM carbonate buffer (pH 9.6) at a concentration of 1 μg/ml. The samples were aliquoted into a 96-well plate (Nunc) in an amount of 200 μg per well, and the plate was coated overnight at 4°C. Then, each well was washed with PBS-T (137 mM NaCl, 2 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, 0.05% Tween 20) three times, blocked with 250 μl of a blocking buffer (1% bovine serum albumin in PBS-T) at room temperature for 1 hr, and washed again with the same PBS-T three times. The standard and test samples were diluted in PBS-T to a predetermined concentration and added to antibody-coated wells, and the plate was incubated at room temperature for 1 hr and washed with PBS-T three times. Thereafter, 2 μg/ml C1q (R&D Systems) was added to the plate and reacted at room temperature for 2 hrs, and the plate was washed with PBS-T six times. 200 μl of a 1:1000 dilution of a human anti-human C1q antibody-peroxidase conjugate (Biogenesis, USA) in the blocking buffer was added to each well and reacted at room temperature for 1 hr. After each wash was washed with PBS-T three times, equal volumes of color reagents A and B (Color A: stabilized peroxide and Color B: stabilized chromogen; DY 999, R&D Systems) were mixed, and 200 μl of the mixture was added to each well, followed by incubation for 30 min. Then, 50 μl of a reaction termination solution, 2 M sulphuric acid, was added to each well. The plate was read using a microplate reader (Molecular Device).

Absorbance of standard and test samples was measured at 450 nm, and the results are given in FIGS. 14 and 15, respectively.

When immunoglobulin subclasses were compared with each other for complement activity in their immunoglobulin Fc fragment, the highest binding affinity to C1q was found in human immunoglobulin IgG1 (Fitzgerald), the next in IgG2 (Fitzgerald) and then IgG4 (Fitzgerald), indicating that there is a difference between subclasses in complement activity. The IVIG used in this test, which is a pool of IgG subclasses, exhibited a C1q binding affinity almost the same as the purified IgG1 because IgG1 amounts to most of the IVIG. Compared to these standards, with respect to changes in binding affinity to C1q by aglycosylation, IgG1 Fc having the strongest complement activity markedly decreased when aglycosylated, IgG4 Fc, known not to induce complement activation, rarely had binding affinity to C1q, indicating that the IgG4 Fc is used as an excellent recombinant carrier with no complement activity (FIG. 14).

To determine whether the carrier maintains its property of having no binding affinity to C1q even after being conjugated to a physiologically active peptide, IFNα alpha-Fc conjugates were prepared using glycosylated Fc, enzymatically deglycosylated Fc, and aglycosylated recombinant Fc as carriers for IFN alpha and were evaluated for their binding affinity to C1q. A glycosylated Fc-coupled IFNα alpha conjugate (IFNα-PEG-Fc: Glycosylated IgG1Fc) maintained a high binding affinity to C1q. In contrast, when interferon alpha was coupled to an Fc deglycosylated using PNGase F and other enzymes, the resulting conjugate (IFNα-PEG-DGFc: Deglycosylated IgG1Fc) displayed a markedly decreased binding affinity to C1q, which was similar to that of the E. coli-derived aglycosylated Fc conjugate. In addition, when the IgG1 moiety of the aglycosylated IgG1 Fc-coupled interferon alpha conjugate (IFNα-PEG-AGFcs1: Aglycosylated IgG1Fc) was exchanged with the IgG4 moiety, the resulting interferon conjugate (IFNα-PEG-FC4 derivative 1: Aglycosylated IgG4Fc) was found to completely lose its binding affinity to C1q. When the IgG1 Fc moiety was exchanged with the IgG4 Fc monomer, the resulting conjugate (IFNα-PEG-FC4 derivative 2: Aglycosylated IgG4Fc) of these results indicate that such forms of the IgG4 Fc fragment are useful as excellent carriers not having the effector functions of antibody fragments (FIG. 15).

INDUSTRIAL APPLICABILITY

As described hereinbefore, the pharmaceutical composition of the present invention greatly increases plasma half-lives of drugs. On the other hand, the protein conjugates overcome the most significant disadvantage of conventional long-acting formulations, decreasing drug titers, thus having blood circulation time and in vivo activity superior to albumin, previously known to be most effective. In addition, the protein conjugates have no risk of inducing immune responses. Due to these advantages, the protein conjugates
are useful for developing long-acting formulations of protein drugs. The long-acting formulations of protein drugs according to the present invention are capable of reducing the patient’s pain from frequent injections, and of maintaining serum concentrations of active polypeptides for a prolonged period of time, thus stably providing pharmaceutical efficacy. Furthermore, the present method of preparing a protein conjugate overcomes disadvantages of fusion protein production by genetic manipulation, including difficult establishment of expression systems, glycosylation different from a native form, immune response induction and limited orientation of protein fusion, low yield due to non-specific reactions, and problems of chemical coupling such as toxicity of chemical compounds used as binders, thereby easily economically providing protein drugs with extended serum half-life and high activity.

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The invention claimed is:

1. A pharmaceutical composition comprising an immunoglobulin Fc fragment as a carrier, wherein said immunoglobulin Fc fragment does not contain the variable regions of the heavy and light chains of the immunoglobulin, and is covalently linked to a drug that is a physiologically active polypeptide through a non-peptide linker.

2. The pharmaceutical composition according to claim 1, wherein the immunoglobulin Fc fragment is aglycosylated.

3. The pharmaceutical composition according to claim 1, wherein the immunoglobulin Fc fragment is composed of one to four domains selected from the group consisting of Cα1, Cα2, Cα3 and Cα4 domains.

4. The pharmaceutical composition according to claim 1, wherein the immunoglobulin Fc fragment further includes a hinge region.

5. The pharmaceutical composition according to claim 1, wherein the immunoglobulin Fc fragment is selected from the group consisting of Fc fragments from IgG, IgA, IgD, IgE, IgM, and combinations and hybrids thereof.

6. The pharmaceutical composition according to claim 1, wherein the immunoglobulin Fc fragment is selected from the group consisting of Fc fragments from IgG1, IgG2, IgG3, IgG4, and combinations and hybrids thereof.

7. The pharmaceutical composition according to claim 1, wherein the immunoglobulin Fc fragment is an IgG4 Fc fragment.

8. The pharmaceutical composition according to claim 1, wherein the immunoglobulin Fc fragment is a human aglycosylated IgG4 Fc fragment.

9. The pharmaceutical composition according to claim 1, wherein said non-peptide linker is selected from the group consisting of polyethylene glycol single polymers, polypropylene glycol single polymers, ethylene glycol propylene glycol copolymers, polyoxyethylated polyols, polyvinyl alcohols, polysaccharides, dextrins, polyvinyl ethyl ethers, biodegradable polymers, lipid polymers, chitin, hyaluronic acids, and combinations thereof.

10. The pharmaceutical composition according to claim 1, wherein the physiologically active polypeptide is selected from the group consisting of hormones, cytokines, enzymes, antibodies, growth factors, transcription regulatory factors, coagulation factors, vaccines, structural proteins, ligand proteins and receptors.

11. The pharmaceutical composition according to claim 10, wherein the physiologically active polypeptide is selected from the group consisting of human growth hormone, growth hormone releasing hormone, growth hormone releasing peptide, interferons, interferon receptors, colony stimulating factors, glucagon-like peptides, G-protein-coupled receptor, interleukin, interleukin receptors, enzymes, interleukin binding proteins, cytokine binding proteins, macrophage activating factor, macrophage peptide, B cell factor, T cell factor, protein A, allergy inhibitor, cell necrosis glycoproteins, immunotoxin, lymphotoxin, tumor necrosis factor, tumor suppressors, metastasis growth factor, alpha-1 antitrypsin, albumin, alpha-lactalbumin, apolipoprotein-E, erythropoietin, highly glycosylated erythropoietin, angiopoietins, hemoglobin, thrombin, thrombin receptor activating peptide, thrombomodulin, factor VII, factor VIIa, factor VIII, factor IX, factor XIII, plasminogen activating factor, fibrin-binding peptide, urokinase, streptokinase, hirudin, protein C, C-reactive protein, renin inhibitor, collagenase inhibitor, superoxide dismutase, leptin, platelet-derived growth factor, epithelial growth factor, epidermal growth factor, angiotensin, angiotensin, bone growth factor, bone stimulating protein, calcitonin, insulin, atriopeptin, cartilage inducing factor, elastin, connective tissue activating factor, tissue factor pathway inhibitor, fibroblast activating hormone, leuteinizing hormone, leuteinizing hormone releasing hormone, nerve growth factors, parathyroid hormone, relaxin, secretin, somatomedin, insulin-like growth factor, adrenocortical hormone, glucagon, cholecystokinin, pancreatic polypeptide, gastrin releasing peptide, corticotropin releasing factor, thyroid stimulating hormone, autotaxin, lactoferrin, myostatin, receptors, receptor antagonists, cell surface antigens, virus derived vaccine antigens, monoclonal antibodies, polyclonal antibodies, and antibody fragments.

12. The pharmaceutical composition according to claim 11, wherein the physiologically active polypeptide is selected from the group consisting of human growth hormone, colony stimulating factor, interferon-alpha, human erythropoietin and Fab' antibody fragment.

13. The pharmaceutical composition according to claim 9, wherein said non-peptide linker is a polyethylene glycol single polymer (PEG).

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